Low-dose antigen-experienced CD4+ T cells display reduced clonal expansion but facilitate an effective memory pool in response to secondary exposure

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Summary

The strength and duration of an antigenic signal at the time of initial stimulation were assumed to affect the development and response of effectors and memory cells to secondary stimulation with the same antigen. To test this assumption, we used T-cell receptor (TCR)-transgenic CD4+ T cells that were stimulated *in vitro* with various antigen doses. The primary effector CD4+ T cells generated in response to low-dose antigen *in vitro* exhibited reduced clonal expansion upon secondary antigenic exposure after adoptive transfer to hosts. However, the magnitude of their contraction was much smaller than both those generated by high-dose antigen stimulation and by naïve CD4+ T cells, resulting in higher numbers of antigen-specific CD4+ T cells remaining until the memory stage. Moreover, secondary effectors and memory cells developed by secondary antigen exposure were not functionally impaired. In hosts given the low-dose antigen-experienced CD4+ T cells, we also observed accelerated recall responses upon injection of antigen-bearing antigen-presenting cells. These results suggest that primary TCR stimulation is important for developing optimal effectors during initial antigen exposure to confer long-lasting memory CD4+ T cells in response to secondary exposure.

Keywords: antigen-experienced CD4+ T cells; antigen doses; memory cells; clonal expansion

Introduction

Upon initial stimulation with antigen-bearing antigen-presenting cells (APC), CD4+ T cells undergo clonal expansion through a series of several rounds of antigen-specific cell division. During clonal expansion, the CD4+ T cells progressively differentiate into specialized effector cells that combat the antigenic insult and memory cells that protect against future exposures to the same antigen.[1,2] Signals transmitted through the T-cell receptor (TCR) and through costimulation receptors are both needed for this proliferation and differentiation of CD4+ T cells.[3,4] Thus, much attention has been focused on the role of antigen in driving this process of CD4+ T-cell proliferation and differentiation. Exposure to antigen for a minimum of 12 hr is required for activation of naive CD4+ T cells and entry into the cell cycle.[5–7] Antigen concentration is also postulated to be important in determining whether CD4+ T cells favour effectors (T helper 1/2; Th1/Th2) and memory cell development.[8–13] Random encounters of variable duration with APC bearing antigen result in the generation of effectors with different fates.[5,14–17] Therefore, the level of TCR signal accumulated by the combination of the duration of TCR engagement and the antigen concentration may determine the progressive differentiation of effectors.

CD4+ memory T cells are potent regulatory cells that can respond rapidly and effectively upon re-encounter with antigen.[18] Several types of evidence have previously supported the finding that effector cells generated from

Abbreviations: ABTS, 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid; AICD, activation-induced cell death; APC, antigen-presenting cell; BrDU, 5-bromo-2′-deoxyuridine; CFA, complete Freund's adjuvant; CFSE, carboxyfluorescein diacetate succinimidyl ester; FBS, Fetal bovine serum; TCR, T-cell receptor; Tg, transgenic.

naïve $CD4^+$ T cells *in vitro* are the direct precursors of memory cells.[19–22] Adoptive transfer of the *in vitro*-generated effector population of $CD4^+$ T cells leads to the generation of memory cells without further antigenic stimulation. In such experimental models, the polarization of the resulting memory cells reflects the polarization of effectors that were introduced,[19] and the spectrum of TCR specificities among *in vivo*-generated memory populations in recipients after adoptive transfer closely parallels those of the primary effector population.[23,24] Moreover, for $CD4^+$ memory T cells generated in response to the transfer of effectors to adoptive hosts, the size of the memory population is directly proportional to the number of effectors that are transferred into recipients, and the duration of *in vitro* activation decide the delineation of memory (central versus effector memory cell) subsets.[25] Such results are mostly focused on the development of $CD4^+$ memory T cells generated from effectors that were adoptively transferred to hosts without further antigenic stimulation. However, less is known about the behaviour of *in vitro*-generated primary effector $CD4^+$ T cells following adoptive transfer upon *in vivo* re-stimulation with the same antigen.

Here we assumed that the strength and duration of antigenic and costimulatory signals during initial stimulation *in vitro* affect the quantity and quality of developing effectors and memory cells in response to *in vivo* re-stimulation with the same antigen. To examine the impact of the initial TCR signal on the quantity and quality of effectors and memory T cells generated from primary effector cells, we used a TCR-transgenic (Tg) model system from which we isolated a homogeneous population of naïve antigen-specific $CD4^+$ T cells. With the use of vital dye and fluorescence-activated cell sorting (FACS) analysis, we found that the restricted stimulation of naïve $CD4^+$ T cells with low and high antigen doses in the presence of polarizing cytokines can result in the development of qualitatively different effector cells, based on the production of polarizing cytokines and the differentiation of $CD4^+$ memory T cells after re-exposure with the same antigen. Therefore, the present results demonstrate the importance of optimal TCR stimulation for developing effectors during initial antigen exposure in order to confer effective long-lasting memory $CD4^+$ T-cell response to secondary exposure.

Materials and methods

Animals

Five- to 6-week old BALB/c mice (Damul Sci. Inc., Daejeon, Korea) and ovalbumin (OVA)-TCR Tg mice (DO11.10 mice, kindly provided by Dr B. S. Kwon, Immunoregulatory Research Center, Ulsan, Korea) were used in this study. The majority of T cells in the DO11.10 mice are $CD4^+$ T cells that express a TCR that recognizes the chicken OVA-derived peptide, $OVA_{323–339}$, presented by $I\text{-}A^d$.[26] This TCR is encoded by transgenes for the $V_{\beta 8.2}/V_{\alpha 13.1}$ chains and can be identified by the anticlonotypic monoclonal antibody (mAb), KJ1.26.[27] DO11.10 severe combined immunodeficiency (SCID) donors were obtained by crossing DO11.10 BALB/c mice for two generations with SCID BALB/c mice; offspring were selected such that almost all (>98%) of the $CD4^+$ T cells were also KJ1.26-positive. BALB/c mice were housed conventionally, and DO11.10 mice were housed in sterile microisolator cages in the animal facility. The investigators adhere to the guidelines set by the Committee on the Care of Laboratory Animal Resources, Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Antibodies and synthetic $OVA_{323–339}$ peptide

The following mAb were obtained from eBioscience (San Diego, CA) and used for FACS analysis and other experiments: phycoerythrin (PE)-anti-CD4 (clone GK1.5), fluorescein isothiocyanate (FITC)-anti-CD25 (clone PC61.5), anti-CD69 (clone H1.2F3), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), and antibromodeoxyuridine (clone PRB-1). Biotinylated KJ1.26 (Caltag Laboratory, Burlingame, CA) and streptavidin–peridinin chlorophyll-a protein (Pharmingen, San Diego, CA) were used to detect OVA-TCR Tg $CD4^+$ T cells. The synthetic peptide of the defined chicken $OVA_{323\text{-}339}$ $H\text{-}2^d$-restricted epitope (ISQAVHAAHAEINEAGR) was synthesized at Peptron Inc. (Daejeon, Korea).

In vitro *generation of antigen-experienced $CD4^+$ T cells*

Spleen and lymph node cells from either OVA-TCR Tg mice or OVA-TCR SCID mice were isolated over nylon-wool columns and treated with a panel of depleting antibodies and complement to enrich the resting $CD4^+$ T cells. The isolated cells routinely consisted of >90% $CD4^+$ T cells. The primary Th1-type $CD4^+$ effector T cells were generated from the enriched naïve $CD4^+$ TCR Tg cell population as described.[28] Briefly, naive $CD4^+$ T cells (5×10^6 cells/ml) were stimulated with syngeneic T-depleted APC obtained from naïve BALB/c mice using a metrizamide gradient (Accurate Chemical Sci., Westbury, NY; analytical grade, 14·5 g added to 100 ml phosphate-buffered saline (PBS), pH 7·2). Th1 effector cells were generated by the addition of 2 ng/ml recombinant murine interleukin (IL)-12 (Peprotech, Rehovot, Israel) and 10 μg/ml anti-IL-4 (clone 11B11) in the presence of either low (0·05 μg/ml) or high (5·0 μg/ml) concentration of $OVA_{323–339}$ peptide. Following a 12-hr stimulation

period, the culture cells were washed to remove $OVA_{323–339}$ peptide. APC were removed with a nylon wool column, and the isolated $CD4^+$ cells were maintained in RPMI medium for 3 days.

Adoptive transfer and immunization

The behaviour of the antigen-experienced $CD4^+$ T cells after secondary exposure was studied by adoptive transfer (via intravenous (i.v.) injection) of the *in vitro*-generated $CD4^+$ effector T cells ($2{\cdot}5 \times 10^6$ $CD4^+$ $KJ1.26^+$ cells/mouse) into non-irradiated normal BALB/c mice. On 1 day after adoptive transfer, recipients were immunized by injection of $OVA_{323–339}$ peptide (20 μg/mouse) emulsified in complete Freund's adjuvant (CFA) into two separate footpad sites. The kinetics of the responses of antigen-experienced $CD4^+$ T cells to secondary exposure were analysed by determining activation markers and the number of OVA-TCR Tg T cells in the popliteal lymph node (LN) following immunization.

Cytokine enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in culture supernatants from OVA-TCR Tg $CD4^+$ T cells stimulated with $OVA_{323–339}$ peptide were determined by standard ELISA. Briefly, 5, 10, and 35 days after immunization, splenocytes and popliteal lymph node cells were obtained from recipients and re-stimulated *in vitro* with syngeneic APC pulsed with $OVA_{323–339}$ peptide (1 μg/ml) for the indicated period (24, 48, and 72 hr). As a polyclonal positive stimulator, a similar number of cells were stimulated with 5 μg of concanavalin A for 48 hr. The culture supernatants were then screened for the presence of IL-2 and interferon-γ (IFN-γ) by ELISA. ELISA plates were incubated with IL-2 and IFN-γ anti-mouse antibody (eBioscience; clone no. JES6-1A12 and R4-6A2, respectively) overnight at 4°. The plates were then washed three times with PBS-0·05% Tween 20 (PBST) and blocked with 3% non-fat dried milk for 2 hr at 37°. The culture supernatant and the appropriate recombinant IL-2 and IFN-γ protein standards (Pharmingen) were added to the plates and incubated overnight at 4°. Biotinylated IL-2 and IFN-γ anti-mouse antibody (eBioscience; clone no. JES6-5H4 and XMG1.2, respectively) were subsequently added and incubated with peroxidase-conjugated streptavidin (Pharmingen) for 1 hr. The colour was then developed by adding a substrate solution 11 mg of 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) in 25 ml of 0·1 M citric acid-25 ml of 0·1 M sodium phosphate−10 μl of hydrogen peroxide). The cytokine concentration in the culture supernatants was determined using an automated ELISA reader and the SOFTmax Pro4.3 program (Spectra MAX340; Molecular Devices, Sunnyvale, CA) compared with two concentrations of standard cytokine protein.

Flow cytometry

OVA-TCR Tg T cells from recipients were evaluated by PE-labelled CD4, biotinylated KJ1.26 antibody, and FITC-labelled CD25, CD44, CD62L, and CD69 antibodies. Briefly, the cells were blocked with heat-inactivated fetal bovine serum (FBS) and washed with PBS containing 1% bovine serum albumin (BSA) and 0·05% NaN_3. The cells were then incubated with the indicated antibody for 45 min at 4°. After washing, the cells were resuspended in PBS and analysed using a FACSCalibur equipped with the CellQuest program (Becton-Dickinson, Mountain View, CA).

Carboxyfluorescein diacetate succinimidyl ester (CFSE) cell labelling and mitotic events. The cell division of OVA-TCR Tg $CD4^+$ T cells primed with antigen in vitro was achieved using CFSE as described.[29] Briefly, enriched $CD4^+$ T cells obtained from DO11.10 BALB/c were washed once in PBS, adjusted to a final concentration of 5×10^7 cells/ml in PBS, and labelled with 5 μM CFSE for 10 min at room temperature. An equal volume of FBS was then added to stop the labelling process, and the cells were washed two times with RPMI containing 10% FBS. The number of cell divisions of CFSE-labelled antigen-experienced $CD4^+$ T cells was estimated as previously described,[29] based on the assumption that the CFSE signal is reduced by half at each cell division.

Detection of 5-bromo-2′-deoxyuridine (BrDU) incorporation. The in vivo proliferation of antigen-specific $CD4^+$ T cells was assessed by BrDU incorporation.[20] Recipients immunized with antigen-pulsed APC were administered BrDU (0.8 mg/ml) daily in their drinking water. After five days, splenocytes were stained with PE-labelled anti-CD4, biotinylated KJ1.26, and PerCP-labelled streptavidin. The cells were then fixed for 20 min in PBS containing 10% formaldehyde and stained for BrDU incorporation according to a published protocol.[20] Briefly, the cells were permeabilized with PBS containing 0·5% saponin and incubated in 1 ml DNase buffer (0·15 mM NaCl, 4·2 mM $MgCl_2$, 4·2 mM $CaCl_2$, 50 Kunitz units/ml DNase I (DN-25; Sigma), pH 5) at 37° for 20 min. The cells were subsequently washed and suspended in PBS containing 1% BSA, 0·05% NaN_3, and 0.5% saponin. The cells were then incubated with FITC-labelled anti-BrDU for 30 min at room temperature. After several washes, the FITC-channel fluorescence of 1000–2000 $CD4^+$ $KJ1.26^+$ cells was measured by flow cytometry.

Statistical analysis

Where specified, the data were analysed for statistical significance using Student's *t*-test. $P < 0{\cdot}05$ was considered statistically significant.

Results

Antigen-experienced CD4+ effector T cells generated *in vitro* with low and high doses

To examine the responses of primary $CD4^+$ effector T cells generated with either low- or high-dose antigen to secondary exposure, we used $OVA_{323–339}$ peptide ($I\text{-}A^d$) specific TCR-Tg $CD4^+$ T cells obtained from naïve DO11.10 BALB/c or DO11.10 SCID mice. The OVA-TCR $CD4^+$ T cells were initially stimulated *in vitro* with either low (0·05 μg/ml) or high (5·0 μg/ml) dose $OVA_{323–339}$ peptide for 12 hr under Th1-type biased conditions (2 ng/ml recombinant murine IL-12 and 10 μg/ml anti-IL-4 clone 11B11). Such stimulated OVA-TCR $CD4^+$ T cells were then analysed to determine the expression of the T-cell surface activation markers such as CD25 (IL-2 receptor a chain, IL-2Rα), CD 69 (very early activation marker), CD44, and CD62L (L-selectin). The 12-hr stimulation of OVA-TCR $CD4^+$ T cells with both low and high antigen doses induced changes in cell size as indicated by the shift of forward scatter profiles to the left (Fig. 1a). The stimulation of OVA-TCR $CD4^+$ T cells with high-dose antigen, in particular, resulted in clear changes in cell size. Similarly the 12-hr stimulation of $CD4^+$ T cells with low and high antigen doses induced activated phenotypic marker profiles for CD25 and CD69 on OVA-TCR $CD4^+$ T cells. The stimulation of OVA-TCR $CD4^+$ T cells by high-dose antigen resulted in clearer activated expression patterns for these activation markers (CD25 and CD69) (Fig. 1a). Also, the expansion of briefly stimulated OVA-TCR $CD4^+$ T cells was examined by labelling the nylon-wool column-enriched T cells that had been rested for 3 days with the fluorescence dye CFSE. As shown by the CFSE profiles, the OVA-TCR $CD4^+$ T cells stimulated *in vitro* with high antigen dose expanded well, whereas no cell division was observed in the naïve and low-dose antigen-stimulated $CD4^+$ T cells during the 3-day resting period (Fig. 1b). These results indicate that the $CD4^+$ T cells stimulated for 12 hr with high-dose antigen *in vitro* were fully activated and were proliferating. However, $CD4^+$ T cells stimulated with low-dose antigen have no proliferative capacity, similar to naïve cells, even though the low-dose antigen-stimulated $CD4^+$ T cells displayed phenotypic markers of activation.

We also observed the number of donor cells in lymphoid and non-lymphoid tissues after adoptive transfer of briefly stimulated $CD4^+$ T cells into normal BALB/c mice. To remove any residual antigen, the OVA-TCR $CD4^+$ T cells stimulated with low- and high-dose antigen for 12 hr were applied to nylon-wool columns, rested for 3 days, and then adoptively transferred into normal mice. On the following day, we determined the number of donor OVA-TCR $CD4^+$ T cells in lymphoid and non-lymphoid tissues. As shown in Fig. 2, decreased numbers of donor cells were observed in the peripheral lymphoid tissues (cervical and popliteal LN), but not in the spleen, in the recipients of $CD4^+$ T cells stimulated with high-dose antigen. In contrast, increased numbers of donor cells were found in non-lymphoid tissue, such as lung, in the recipients of high-dose antigen-stimulated $CD4^+$ T cells. Higher numbers of both naïve and low-dose antigen-stimulated donor cells were observed in peripheral lymphoid tissues compared with high-dose antigen-stimulated $CD4^+$ T cells. Moreover, there were no significant differences between naïve and low-dose antigen-stimulated OVA-TCR $CD4^+$ T cells found in both lymphoid and non-lymphoid tissues (Fig. 2). This result indicates that the $CD4^+$ T cells stimulated with low- and high-dose antigen have different capacities for migration into lymphoid and non-lymphoid tissues.

In vivo clonal expansion potential and memory cells of antigen-experienced CD4+ T cells after secondary exposure to antigen

Primary $CD4^+$ effector T cells previously experienced with low and high antigen doses were assumed to respond alternatively to secondary exposure of antigen. In order to test this assumption, we used *in vivo* secondary exposure of antigen after adoptive transfer of briefly stimulated OVA-TCR $CD4^+$ T cells into normal BALB/c mice. OVA-TCR $CD4^+$ T cells stimulated *in vitro* with low and high antigen doses for 12 hr were applied to nylon-wool columns to remove further antigenic stimulation, rested for 3 days, and then adoptively transferred into normal mice. On next day, the recipients were immunized via the hind footpad with $OVA_{323–339}$ peptide emulsified in CFA. On 5, 10, and 35 days postimmunization (p.i), the number of OVA-TCR $CD4^+$ T cells and immune responses were kinetically measured in popliteal LN. As shown in Fig. 3(a and b), the peak of the response for both low- and high-antigen dose-stimulated donor cells appeared to be day 5. By day 10 after immunization, the number of OVA-TCR $CD4^+$ T cells had subsequently decreased in popliteal LN. Also, naive donors, which were not stimulated with antigen, exhibited similar response patterns to the *in vivo* exposure of antigen. However, there were differences in the magnitude of the expansion and contraction of OVA-TCR $CD4^+$ T cells depending on the antigen dose used for the primary *in vitro* exposure (Fig. 3a and b). Five days p.i., $CD4^+$ T cells previously stimulated with high-dose antigen for 12 hr expanded maximally with approximately 15-fold increase, whereas low antigen dose-stimulated OVA-TCR $CD4^+$ T cells displayed the least expansion in response to *in vivo* secondary exposure of antigen. Naive donors exhibited a median magnitude of expansion to antigen immunization. Conversely, after the fifth day

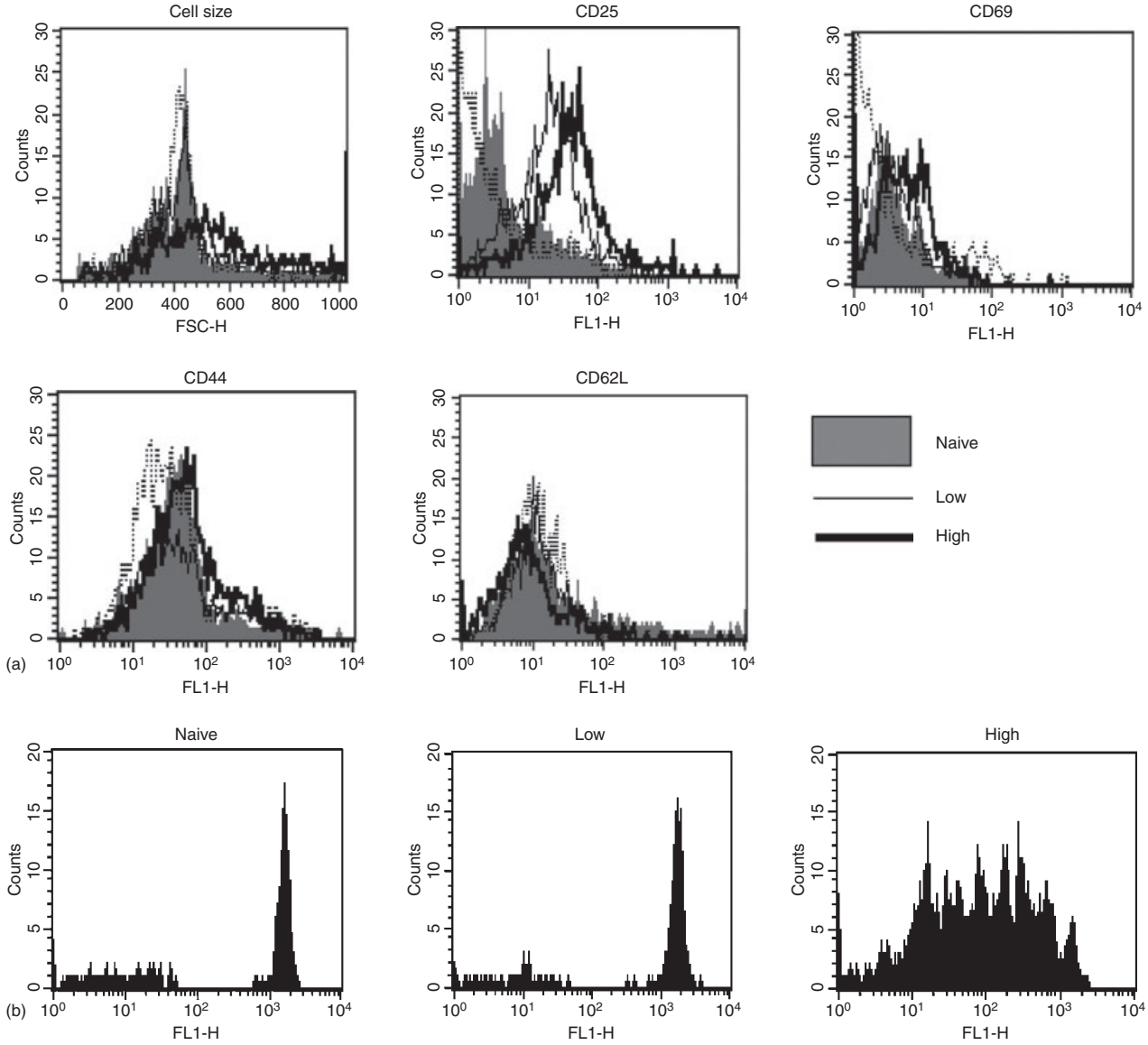

Figure 1. (a) Activation phenotypes of antigen-experienced $CD4^+$ T cells generated *in vitro* with different antigen doses. Naive OVA-TCR $CD4^+$ T cells enriched by passage over a nylon-wool column and treated with depleting antibodies and complement were stimulated with APC in the presence of low (0·05 μg/ml) and high (5·0 μg/ml) concentrations of $OVA_{323–339}$ peptide with the Th1-polarized cytokines. Following the 12-hr antigen stimulation, the expression profiles of activation phenotypic markers (CD25, CD69, CD44, and CD62L) of $CD4^+$ $KJ1.26^+$ T cells were determined by FACS analysis. Dot lines represent activation phenotypes of naïve antigen-specific $CD4^+$ T cells that did not pass nylon-wool column. (b) Proliferation potential of antigen-experienced $CD4^+$ T cells generated *in vitro* with different antigen doses. Following the 12-hr antigen stimulation, primary $CD4^+$ T effector cells were applied to a nylon-wool column to remove further antigenic stimulation and stained with the fluorescence dye CFSE. CFSE-labelled cells were then rested for 3 days. The proliferation of antigen-experienced $CD4^+$ T cells was estimated, based on the assumption that the CFSE signal is reduced by half at each cell division. The histograms are based on a gate specific for the antigen-specific $CD4^+$ T cells ($KJ1.26^+$ $CD4^+$).

p.i., the magnitude of contraction appeared to reverse, because the least number of OVA-TCR $CD4^+$ T cells previously experienced with high-dose antigen were detected in draining LN at 10 days p.i. These OVA-TCR $CD4^+$ T cells continuously decreased until 35 days p.i., resulting in generating recipients that contained comparable numbers of initial OVA-TCR $CD4^+$ T cells. However, the recipients that received low-dose antigen-experienced OVA-TCR $CD4^+$ T cells underwent the smallest contraction after secondary exposure to antigen, resulting in many more antigen-specific $CD4^+$ T cells remaining in the LN than in recipients given high-dose antigen-stimulated OVA-TCR $CD4^+$ T cells (Fig. 3b). Such results are clarified when the expansion and contraction of antigen-

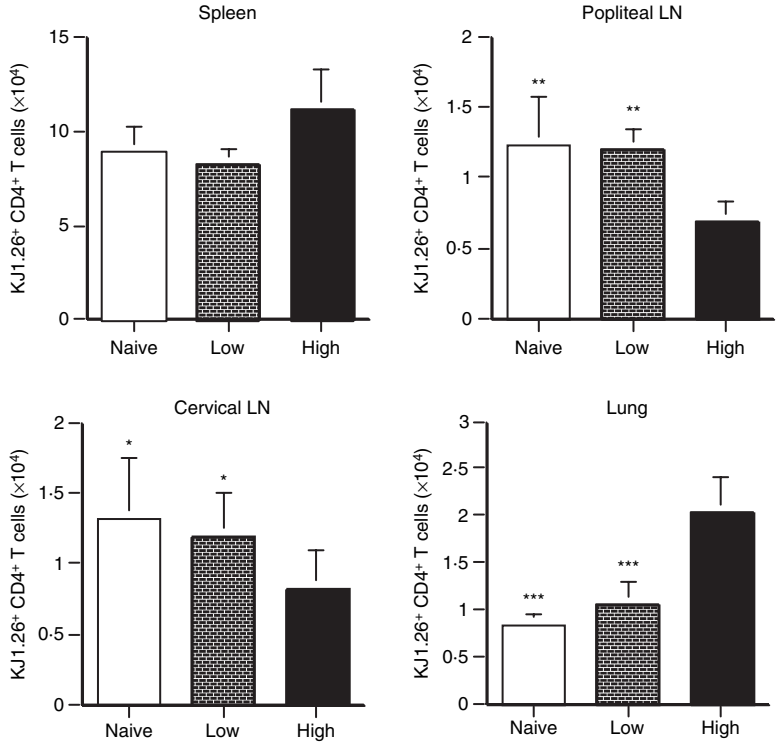

Figure 2. The migration of antigen-experienced CD4$^+$ T cells generated *in vitro* with the different antigen doses into lymphoid and non-lymphoid tissues. Antigen-experienced CD4$^+$ T cells generated *in vitro* with low (0·05 μg/ml) and high (5·0 μg/ml) concentration of $OVA_{323–339}$ peptide were rested for 3 days, and then adoptively transferred into hosts via i.v. injection ($2·5 \times 10^6$ CD4$^+$ KJ1.26$^+$ cells/mouse). On the following day, the number of donor OVA-TCR CD4$^+$ T cells was determined in lymphoid and non-lymphoid tissues by staining with specific antibodies and FACS analysis. Values represent the absolute number of KJ1.26 $^+$ CD4$^+$ T cells and are shown as the mean ± SD of three to five replicates per experiment. *Statistically significant between high-dose antigen-stimulated and the indicated groups ($P < 0·05$). **Statistically significant between high-dose antigen-stimulated and the indicated groups ($P < 0·01$). ***Statistically significant between high-dose antigen-stimulated and the indicated groups ($P < 0·001$).

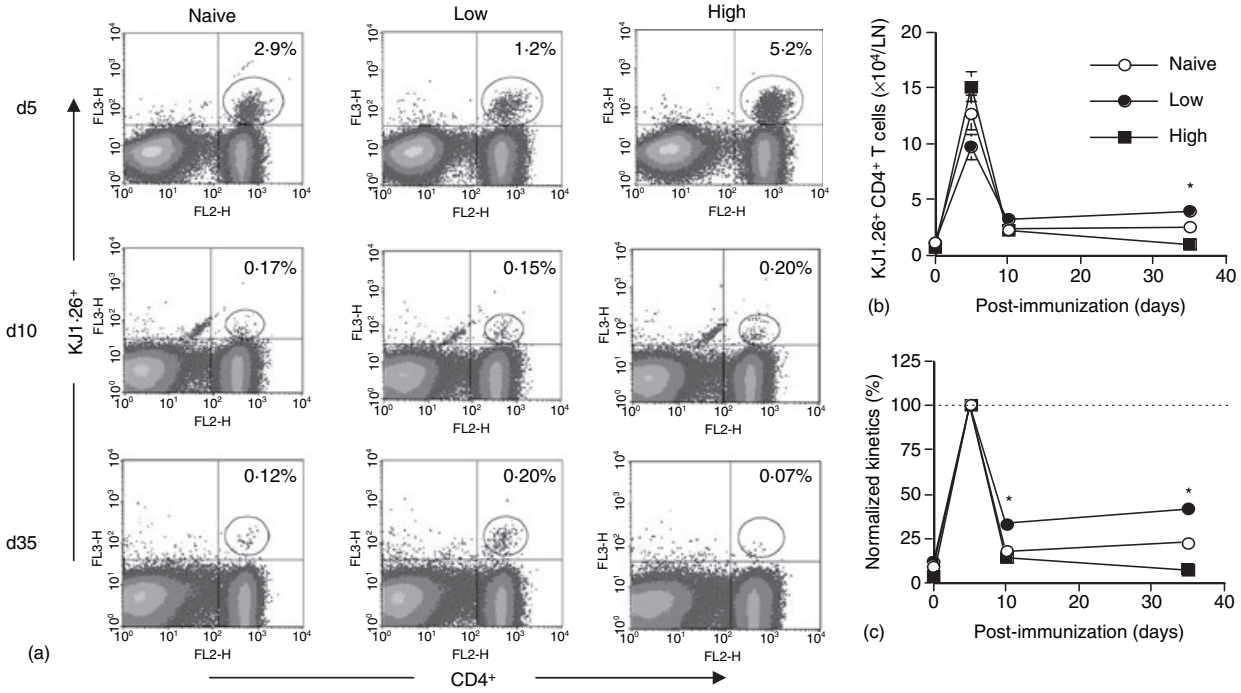

Figure 3. The behaviour of antigen-experienced CD4$^+$ T cells generated *in vitro* with different antigen doses in response to secondary antigenic exposure. Antigen-experienced CD4$^+$ T cells generated *in vitro* with low (0·05 μg/ml) and high (5·0 μg/ml) concentrations of $OVA_{323–339}$ peptide were rested for 3 days and then adoptively transferred into normal BALB/c mice via i.v. injection ($2·5 \times 10^6$ CD4$^+$ KJ1.26$^+$ cells/mouse). On the following day, the recipients were immunized via the hind footpad with $OVA_{323–339}$ peptide (20 μg/mouse) emulsified with CFA. On 5, 10, and 35 days p.i., the behaviour of antigen-experienced CD4$^+$ T cells was examined by staining with specific antibodies and FACS analysis in popliteal LNs. (a) The percentage of KJ1.26 $^+$ CD4$^+$ T cells from representative recipients in popliteal LNs. (b) Total number of KJ1.26$^+$ CD4$^+$ T cells in the popliteal LNs of three to five recipients. (c) Number of KJ1.26$^+$ CD4$^+$ T cells in the popliteal LNs of three to five recipients normalized to the peak of expanded response (5 days p.i). Data are shown as the mean ± SD of three to five recipients per experiment. *Statistically significant between low-dose and high-dose antigen-experienced groups ($P < 0·05$).

specific CD4$^+$ T cells are normalized to the peak response (at day 5) (Fig. 3c). Thus, the recipients given low-dose antigen-experienced OVA-TCR CD4$^+$ T cells contained ~35% of the expanded antigen-specific CD4$^+$ T cells at 35 days p.i., whereas only ~5% of the antigen-specific CD4$^+$ T cells expanded by secondary exposure were detected in draining LN of mice that received high-dose antigen-stimulated OVA-TCR CD4$^+$ T cells. Therefore, although there was minimal accumulation of low-dose antigen-experienced OVA-TCR CD4$^+$ T cells in the draining LN after *in vivo* secondary exposure, the magnitude of their contraction was much smaller, resulting in higher numbers of antigen-specific CD4$^+$ T cells remaining until the memory stage.

Cytokine production and phenotypes of antigen-experienced CD4$^+$ T cells after secondary exposure of antigen

The CD4$^+$ T cells experienced with low-dose antigen may elicit impaired function after secondary exposure,[30] even though the recipients of such low-dose antigen-stimulated OVA-TCR CD4$^+$ T cells contained higher numbers of antigen-specific CD4$^+$ T cells than recipients of high-dose peptide-experienced OVA-TCR CD4$^+$ T cells. Because the immune function of CD4$^+$ T cells are mediated, in large part, by the effector cytokines that they produce,[31] we examined whether OVA-TCR CD4$^+$ T cells previously experienced with low- and high-dose antigen could produce the cytokines IFN-γ and IL-2 after secondary exposure to antigen *in vivo*. Cytokine production by the draining LN cells was determined on 5, 10, and 35 days p.i. in the recipients of antigen-experienced CD4$^+$ T cells. The LN cells of the recipients of both types of experienced OVA-TCR CD4$^+$ T cells had the capacity to secrete cytokines following brief peptide stimulation (Fig. 4). Interestingly, the pattern of cytokine production from the LN cells was kinetically similar to that of the number of OVA-TCR CD4$^+$ T cells detected in the draining LN cells of the recipients. Thus at 5 days p.i., the recipients of OVA-TCR CD4$^+$ T cells experienced with low-dose antigen produced the least amount of IFN-γ and IL-2, but the profile of cytokine production was reversed at 10 and 35 days p.i. Therefore, the amount of cytokine produced from the draining LN cells of the recipients given antigen-experienced OVA-TCR CD4$^+$ T cells was closely related to the number of antigen-specific CD4$^+$ T cells detected. Also, when the phenotype of OVA-TCR CD4$^+$ T

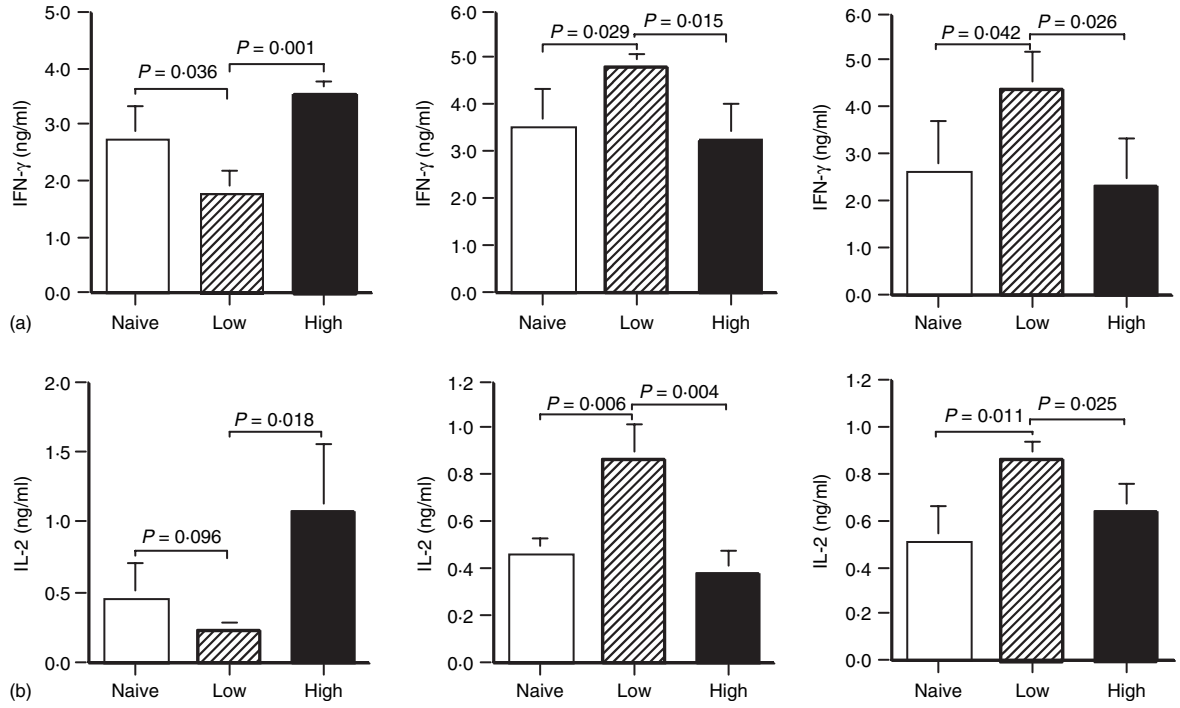

Figure 4. The production of polarized cytokine IFN-γ and IL-2 by antigen-experienced CD4$^+$ T cells generated *in vitro* with different antigen doses. Antigen-experienced CD4$^+$ T cells generated *in vitro* with low (0·05 μg/ml) and high (5·0 μg/ml) concentrations of $OVA_{323–339}$ peptide were rested for 3 days and then adoptively transferred into normal BALB/c mice via i.v. injection ($2·5 \times 10^6$ CD4$^+$ KJ1.26$^+$ cells/mouse). On the following day, the recipients were immunized via the hind footpad with $OVA_{323–339}$ peptide (20 μg/mouse) emulsified with CFA. On 5, 10, and 35 days p.i., the cytokines secreted by antigen-specific CD4$^+$ T cells in the total popliteal LN cells were determined by a standard ELISA after brief stimulation with $OVA_{323–339}$ peptide (1 μg/ml). Data are shown as the mean ± SD of three to five recipients per experiment. *P*-values in graphs were statistically calculated by Student's *t*-test.

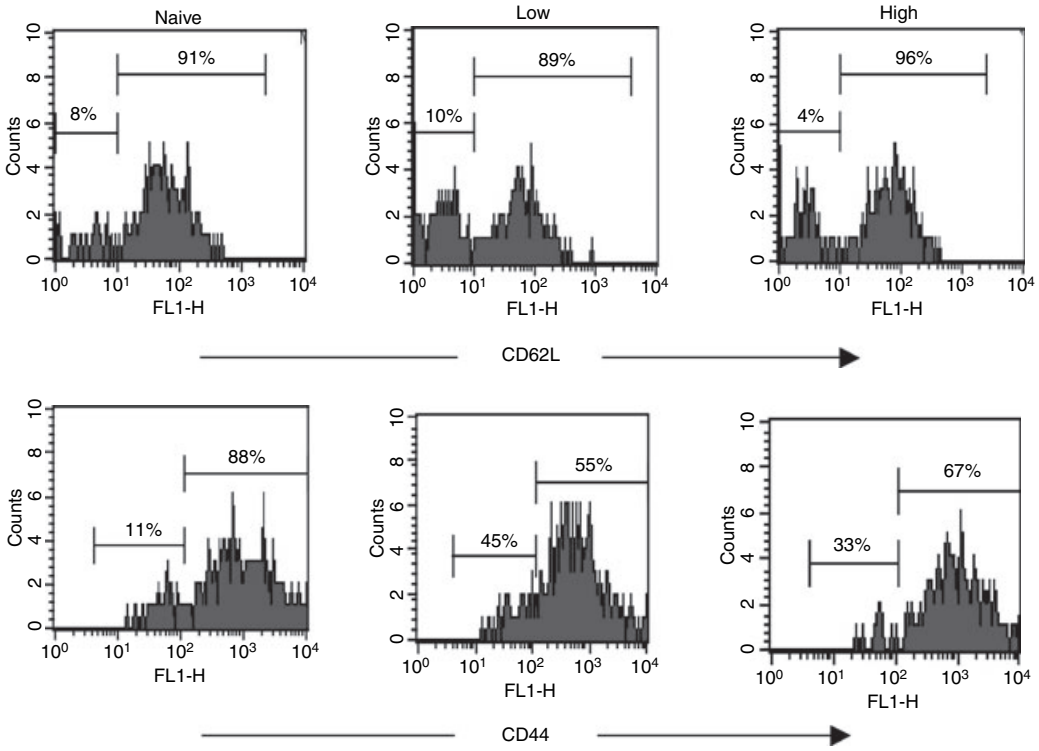

Figure 5. Phenotypes of antigen-specific CD4+ T memory cells developed by primary effector cells in response to secondary antigenic exposure. Antigen-experienced CD4+ T cells generated *in vitro* with low (0·05 μg/ml) and high (5·0 μg/ml) concentrations of $OVA_{323–339}$ peptide were rested for 3 days and then adoptively transferred into normal BALB/c mice via i.v. injection ($2·5 \times 10^6$ CD4+ KJ1.26+ cells/mouse). On the next day, the recipients were immunized via the hind footpad with $OVA_{323–339}$ peptide (20 μg/mouse) emulsified with CFA. On 35 days p.i., the expression of phenotypic markers (CD44 and CD62L) of CD4+ KJ1.26 + T cells obtained from the popliteal LNs was determined by FACS analysis. The histograms based on a gate specific for the antigen-specific CD4+ T cells (KJ1.26+ CD4+) are representative of three to five recipients per experiment and values in histograms are the mean percent of experiments.

cells was analysed 35 days p.i. (memory stage), the majority of antigen-specific CD4+ T cells exhibited central memory-like phenotypes ($CD62L^{high}$) in all recipients tested (Fig. 5). These results indicate that antigen-experienced CD4+ T cells can differentiate into fully functional memory cells after secondary exposure. Moreover, it was of interest that the antigen-specific CD4+ T cells experienced with low-dose antigen retained higher numbers of memory cells after secondary exposure as a result of reduced contraction.

Accelerated recall responses of memory cells generated from low-dose antigen-experienced CD4+ T cells after secondary exposure

One of the hallmark features of memory CD4+ T cells is the recall response mediated by rapid clonal expansion and large quantities of cytokine produced upon re-stimulation with antigen-pulsed APC.[18,25] Here we investigated the recall responses of antigen-specific memory CD4+ T cells generated from CD4+ T cells previously experienced with low- and high-dose peptide. For analysis, the briefly stimulated OVA-TCR CD4+ T cells were adoptively transferred to normal BALB/c mice. The recipients were then immunized with $OVA_{323–339}$ peptide emulsified in CFA. On day 60 p.i., the recipients were injected with $OVA_{323–339}$ peptide-pulsed APC via tail vein, and the frequency of OVA-TCR CD4+ T cells and immune responses were then analysed five days after re-stimulation with antigen/APC. As shown in Fig. 6(a), the recipients of CD4+ T cells experienced with low-dose antigen accumulated greater numbers of OVA-TCR CD4+ T cells in response to re-stimulation with antigen/APC. Also, the recipients of low-dose antigen-experienced CD4+ T cells contained higher numbers of OVA-TCR CD4+ T cells in their spleens 5 days after *in vivo* re-stimulation with antigen/APC (Fig. 6b).

To enumerate antigen-specific CD4+ T cells proliferating in the recall response, the recipients injected with antigen/APC were also administered BrDU (0·8 mg/ml) in the drinking water (day 0). On day 5, BrDU incorporation into antigen-specific CD4+ T cells was determined by FACS analysis. The OVA-TCR CD4+ T cells of the recipients of low-dose antigen-experienced CD4+ T cells

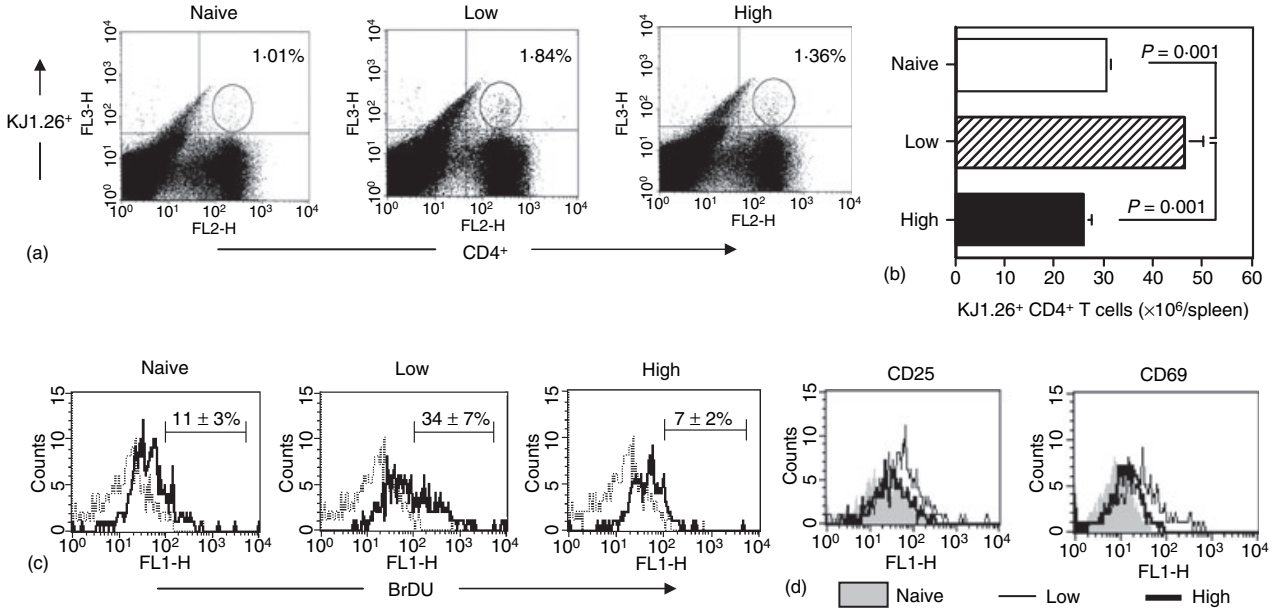

Figure 6. Recall responses of antigen-specific $CD4^+$ T memory cells developed by primary effector cells generated *in vitro* with the different antigen doses in response to secondary antigen exposure. Antigen-experienced $CD4^+$ T cells generated *in vitro* with low (0·05 μg/ml) and high (5·0 μg/ml) concentrations of $OVA_{323–339}$ peptide were rested for 3 days and then adoptively transferred into normal BALB/c mice via i.v. injection ($2·5 \times 10^6$ $CD4^+$ $KJ1.26^+$ cells/mouse). On the next day, the recipients were immunized via the hind footpad with $OVA_{323–339}$ peptide (20 μg/mouse) emulsified with CFA. On 60 days p.i., the recipients were injected with $OVA_{323–339}$ peptide-pulsed APC via tail vein ($2·0 \times 10^7$ cells/mouse) and then the recall responses of antigen-specific $CD4^+$ T cells were examined 5 days after injection of antigen/APCs. (a) The percentage of $KJ1.26^+$ $CD4^+$ T cells in the spleen of representative recipients re-challenged with antigen/APC. (b) Total number of $KJ1.26^+$ $CD4^+$ T cells expanded with antigen/APC in the spleen of three to five recipients. *P*-values in graph were statistically calculated by Student's *t*-test. (c) Enumeration of proliferating antigen-specific $CD4^+$ T cells during recall response with antigen/APC. The recipients injected with antigen/APC were administered BrDU (0·8 mg/ml) in the drinking water. On day 5, BrDU incorporation of antigen-specific $CD4^+$ $KJ1.26^+$ T cells was determined by FACS analysis. The histograms based on a gate specific for the antigen-specific $CD4^+$ T cells ($KJ1.26^+$ $CD4^+$) are representative of three to five recipients and values in histograms are the mean ± SD of each experiment. The dot-lines of histogram denote isotype control. (d) The expression profiles of early activation markers (CD25 and CD69) on the cell surface of antigen-specific $CD4^+$ T cells. The histograms are representative of three to five recipients.

exhibited enhanced incorporation of BrDU (around 34%) compared to recipients given naïve and high-dose antigen-experienced $CD4^+$ T cells (Fig. 6c). CD25 and CD69 are prominently cited markers of recent antigenic stimulation.[32,33] Thus, we also focused on the expression of these two molecules on OVA-TCR $CD4^+$ T cells after the recall response. As shown in Fig. 6(d), the OVA-TCR $CD4^+$ T cells of the recipients that received low-dose antigen-experienced $CD4^+$ T cells exhibited increased expression of both activation markers 5 days after injection of APC pulsed with $OVA_{323–339}$ peptide. These results indicate that memory $CD4^+$ T cells in the recipients of low-dose antigen-experienced $CD4^+$ T cells respond to the recall antigen more rapidly than donor cells in other recipients. We also determined the quantities of the cytokines IFN-γ and IL-2 produced from splenocytes of the recipients given antigen-experienced $CD4^+$ T cells. Consistent with rapid recall responses upon re-stimulation with antigen/APC, the recipients of low-dose antigen-experienced OVA-TCR $CD4^+$ T cells produced higher levels of cytokine production than other recipients following brief $OVA_{323–339}$ peptide stimulation of splenocytes (Fig. 7a and b). Taken together, these results indicate that the recipients of low-dose antigen-experienced $CD4^+$ T cells effectively generated memory $CD4^+$ T cells after secondary exposure, resulting in improved recall responses to the introduced antigen.

Discussion

In this study, the strength and duration of antigenic and costimulatory signal during the initial stimulation *in vitro* was assumed to affect the quantity and quality of developing effector and memory cells upon secondary antigenic exposure. Because we know that several factors including antigen levels, duration of TCR stimulation, costimulatory signals, and growth factors each have major impact on the generation of primary CD4 effector cells,[34,35] our study focused on the antigen dose applied during primary stimulation for a limited duration of TCR

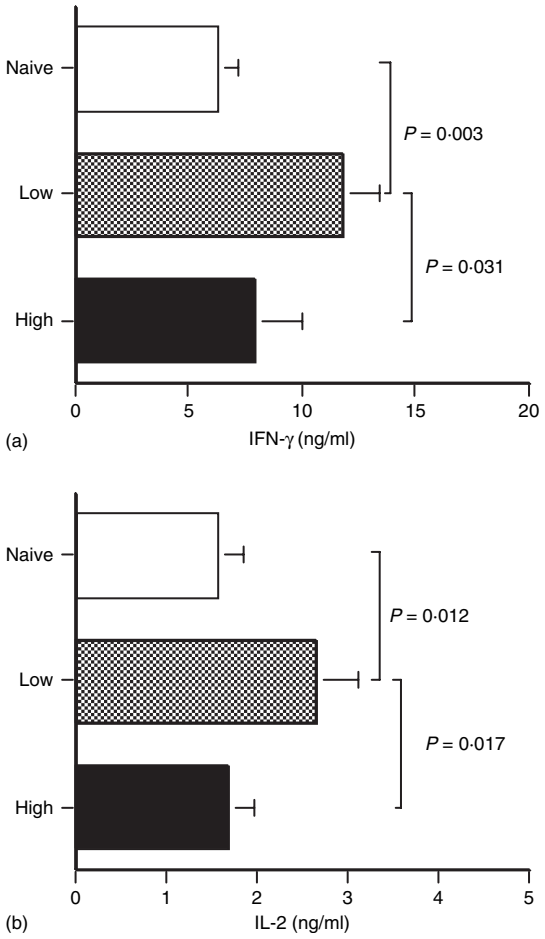

Figure 7. The production of polarized cytokines IFN-γ and IL-2 during recall response by antigen-specific $CD4^+$ T memory cells developed by primary effector cells generated *in vitro* with different antigen doses. Antigen-experienced $CD4^+$ T cells generated *in vitro* with low (0·05 μg/ml) and high (5·0 μg/ml) concentrations of $OVA_{323–339}$ peptide were rested for 3 days and then adoptively transferred into normal BALB/c mice via i.v. injection ($2·5 \times 10^6$ $CD4^+$ $KJ1.26^+$ cells/mouse). On the next day, the recipients were immunized via the hind footpad with $OVA_{323–339}$ peptide (20 μg/mouse) emulsified with CFA. On At 60 days p.i., the cytokine levels were determined by a standard ELISA in the splenocytes of recipients 5 days after re-challenging with antigen/APC. Data are shown as the mean ± SD of three to five recipients per experiment. *P*-values in graphs were statistically calculated by Student's *t*-test.

activation. Here, we noted that, after adoptive transfer to hosts, primary $CD4^+$ effector T cells generated *in vitro* with low-dose antigen exhibited reduced clonal expansion in the draining LN in response to secondary antigenic stimulation. However, the magnitude of their subsequent contraction was much smaller than that of high-dose antigen-stimulated $CD4^+$ T cells so that higher numbers of antigen-specific $CD4^+$ T cells remained until the memory stage. The secondary effector cells differentiated from high-dose antigen-experienced $CD4^+$ T cells were rapidly exhausted such that fewer memory cells remained. Our demonstration is consistent with the finding that prolonged antigen-presentation resulted in a robust expansion of highly differentiated effector, which contracted to a small number of memory T cells.[17] Similarly, it has been reported that the limited activation of $CD4^+$ T cells showed a modest expansion of effectors, but provided persistent memory cells with high efficiency.[17] Therefore, these results suggest the importance of primary TCR stimulation for developing effectors during initial antigen stimulation that confer effective long-lasting memory $CD4^+$ T cells after secondary exposure.

The direct injection of antigen with CFA was used to generate primary $CD4^+$ effector cells in terms of *in vivo* antigen-experienced $CD4^+$ T cells.[36] However, with direct antigen injection, we can not validate that all naïve $CD4^+$ T cells differentiate into primary effector cells as a result of encounters between $CD4^+$ T cells and antigen-bearing APC. We therefore used *in vitro* stimulation with different antigen doses so that naïve $CD4^+$ T cells in the reactions could be uniformly activated. Using *in vivo*-generated antigen-experienced $CD4^+$ T cells, Jenkins' group claimed that reduced clonal expansion potential of antigen-experienced $CD4^+$ T cells was imposed by factors present in the immune environment of hosts.[36] Furthermore, they demonstrated that the clonal expansion potential could be recovered after adoptive transfer of antigen-experienced $CD4^+$ T cells into new naïve mice. Similarly, after allowing them to rest for 3 days, we adoptively transferred primary $CD4^+$ effector cells generated *in vitro* with antigen into hosts. It is conceivable that 3 days of resting in the absence of further antigenic stimulation induced the clonal expansion potential recovery, at least in case of high-dose antigen-stimulated $CD4^+$ T cells. However, after repeated stimulation, low-dose antigen-experienced $CD4^+$ T cells exhibit reduced magnitude of both expansion and contraction. In particular, the number of antigen-specific $CD4^+$ T cells was highly maintained in the recipients of low-dose antigen-stimulated $CD4^+$ T cells. Consequently, ~35% of the expanded antigen-specific $CD4^+$ T cells remained at 35 days p.i. Presumably, enhanced resistance to apoptosis induced by secondary antigenic stimulation may be responsible for this maintenance of antigen-specific $CD4^+$ T cells in the recipients given low-dose antigen-stimulated $CD4^+$ T cells. The survival signals, such as the expression patterns of IL-7R expression[37] and antiapoptotic factors,[38] should be investigated in future studies.

To study the differentiation of naïve $CD4^+$ T cells in polarized effectors and memory cells,[19–21] several groups have developed adoptive transfer techniques for *in vitro*-generated $CD4^+$ T cells. Although many of the signals required for effector differentiation are known, much attention has been focused on the role of the antigen in driving the proliferation and differentiation of $CD4^+$ T

cells. Moreover, antigen concentration may be related to both the extent and duration of TCR engagement, and the duration of persistent antigenic stimulation may determine if differentiation of naïve CD4 T cells favours effectors with different fates.[5,10–15] Thus, the initial programming of $CD4^+$ T cells to become effector (Th1/Th2) and memory cells is likely to differ depending on the extent of TCR stimulation and the cytokine environment.[5,10–15] We here chose a minimal incubation time (12 hr) for activation of naïve $CD4^+$ T cells and entry into the cell cycle occurred in the presence of polarized cytokines with low- or high-dose antigen. According to the expression profiles of the T-cell surface activation markers, $CD4^+$ T cells stimulated *in vitro* with low- and high-dose antigen were activated. In particular, up-regulation of CD25 and CD69 on the surface of $CD4^+$ T cells stimulated with antigen were clearly observed. Other reports have shown that both CD25 and CD69 are increased before cell division.[32,33] Following 12 hr-stimulation with high-dose antigen, $CD4^+$ T cells eventually divided during a 3-day resting period without further antigenic stimulation, whereas little cell division was observed in $CD4^+$ T cells stimulated with low-dose antigen. Moreover, it was observed that $CD4^+$ T cells stimulated with low- and high-dose antigen had different capacities of migration into lymphoid and non-lymphoid tissues. However, the different homing patterns were not correlated to CD62L and CD44 expression on stimulated $CD4^+$ T cells. This observation may be supported by the recent finding that $CD62L^-$ effector and memory $CD8^+$ T cells enter LNs to decrease antigen presentation of DCs.[39] Also, $CD4^+$ T cells stimulated with high-dose antigen for 12 hr secreted greater amounts of IL-2 in culture supernatants (data not shown). This suggests that the low antigen dose we used to generate primary effector cells is suboptimal for inducing proliferation and production of the growth factor IL-2. However, it is possible that IL-2 produced from high-dose antigen-stimulated $CD4^+$ T cells drives cell division during the 3-day resting period and conversely makes antigen-experienced $CD4^+$ T cells susceptible to activation-induced cell death (AICD).[40,41] We also showed that effectors and memory cells developing from low-dose antigen-stimulated $CD4^+$ T cells were not impaired functionally following secondary antigen exposure, as determined by polarized cytokine production and activation phenotype markers. These data suggest that a factor(s) and/or microenvironment driven by the initial TCR stimulation with the different antigen doses may program the characteristics of secondary responses of antigen-experienced $CD4^+$ T cells.

The features of memory $CD4^+$ T cells include a rapid response that involves cytokine production and cell division.[18,25] In experiments to measure recall responses, we observed an extended accumulation of antigen-specific $CD4^+$ T cells upon re-challenge with antigen (APC/antigen) in the recipients of low-dose antigen-stimulated $CD4^+$ T cells. The recipients of low-dose antigen-stimulated $CD4^+$ T cells also consistently exhibited greater numbers of proliferating $CD4^+$ T cells and greater cytokine production by antigen-specific $CD4^+$ T cells. Considering that booster immunizations are needed in many vaccination protocols against infectious diseases,[42,43] our results suggest that the generation of optimal effector $CD4^+$ T cells during initial TCR stimulation has a major impact on the development of the highest number of secondary memory cells. Therefore, our observation has important implications for understanding the generation of primary effector cells in prime-boost vaccinations and may offer improved strategies for the development of long-lasting memory T cells.

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References

1 Swain SL, Croft M, Dubey C, Haynes L, Rogers P, Zhang X, Bradley LM. From naive to memory T cells. *Immunol Rev* 1996; **150**:143–67.

2 Sprent J, Surh CD. T cell memory. *Annu Rev Immunol* 2002; **20**:551–79.

3 Davis MM, Boniface JJ, Reich Z, Lyons D, Hampl J, Arden B, Chien Y. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* 1998; **16**:523–44.

4 van der Merwe PA, Davis SJ. Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol* 2003; **21**:659–84.

5 Iezzi G, Karjalainen K, Lanzavecchia A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 1998; **8**:89–95.

6 Lanzavecchia A, Lezzi G, Viola A. From TCR engagement to T cell activation. a kinetic view of T cell behavior. *Cell* 1999; **96**: 1–4.

7 Jelley-Gibbs DM, Lepak NM, Yen M, Swain SL. Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. *J Immunol* 2000; **165**:5017–26.

8 Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med* 1995; **182**:1579–84.

9 Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J Exp Med* 1995; **182**:1591–6.

10 Farber DL. Differential TCR signaling and the generation of memory T cells. *J Immunol* 1998; **160**:535–9.
11 Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation. impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol* 2000; **1**:311–6.
12 Iezzi G, Scheidegger D, Lanzavecchia A. Migration and function of antigen-primed nonpolarized T lymphocytes *in vivo*. *J Exp Med* 2001; **193**:987–93.
13 Langenkamp A, Casorati G, Garavaglia C, Dellabona P, Lanzavecchia A, Sallusto F. T cell priming by dendritic cells. thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur J Immunol* 2002; **32**:2046–54.
14 Gett AV, Sallusto F, Lanzavecchia A, Geginat J. T cell fitness determined by signal strength. *Nat Immunol* 2003; **4**:355–60.
15 Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. *Nat Rev Immunol* 2002; **2**:982–7.
16 Corbin GA, Harty JT. Duration of infection and antigen display have minimal influence on the kinetics of the CD4+ T cell response to *Listeria monocytogenes* infection. *J Immunol* 2004; **173**:5679–87.
17 Jelley-Gibbs DM, Brown DM, Dibble JP, Haynes L, Eaton SM, Swain SL. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J Exp Med* 2005; **202**:697–706.
18 Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol* 2003; **4**:835–42.
19 Swain SL, Hu H, Huston G. Class II-independent generation of CD4 memory T cells from effectors. *Science* 1999; **286**:1381–3.
20 Hu H, Huston G, Duso D, Lepak N, Roman E, Swain SL. CD4 (+) T cell effectors can become memory cells with high efficiency and without further division. *Nat Immunol* 2001; **2**:705–10.
21 Rogers PR, Dubey C, Swain SL. Qualitative changes accompany memory T cell generation. faster, more effective responses at lower doses of antigen. *J Immunol* 2000; **164**:2338–46.
22 Moulton VR, Bushar ND, Leeser DB, Patke DS, Farber DL. Divergent generation of heterogeneous memory CD4 T cells. *J Immunol* 2006; **177**:869–76.
23 Blattman JN, Sourdive DJ, Murali-Krishna K, Ahmed R, Altman JD. Evolution of the T cell repertoire during primary, memory, and recall responses to viral infection. *J Immunol* 2000; **165**:6081–90.
24 Varga SM, Welsh RM. Stability of virus-specific CD4+ T cell frequencies from acute infection into long term memory. *J Immunol* 1998; **161**:367–74.
25 Swain SL. Regulation of the generation and maintenance of T-cell memory: a direct, default pathway from effectors to memory cells. *Microbes Infect* 2003; **5**:213–9.
26 Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+ CD8+ TCR^{lo} thymocytes *in vivo*. *Science* 1990; **250**:1720–3.
27 Haskins K, Kubo R, White J, Pigeon M, Kappler J, Marrack P. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J Exp Med* 1983; **157**:1149–69.
28 Swain SL. Generation and *in vivo* persistence of polarized Th1 and Th2 memory cells. *Immunity* 1994; **1**:543–52.
29 Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J Clin Invest* 1997; **100**:3173–83.
30 Schwartz RH. T cell anergy. *Annu Rev Immunol* 2003; **21**:305–34.
31 O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998; **8**:275–83.
32 Bird JJ, Brown DR, Mullen AC *et al.* Helper T cell differentiation is controlled by the cell cycle. *Immunity* 1998; **9**:229–37.
33 Lee WT, Pasos G, Cecchini L, Mittler JN. Continued antigen stimulation is not required during CD4 (+) T cell clonal expansion. *J Immunol* 2002; **168**:1682–9.
34 Rogers PR, Croft M. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* 1999; **163**:1205–13.
35 Merica R, Khoruts A, Pape KA, Reinhardt RL, Jenkins MK. Antigen-experienced CD4 T cells display a reduced capacity for clonal expansion in vivo that is imposed by factors present in the immune host. *J Immunol* 2000; **164**:4551–7.
36 Seder RA. High-dose IL-2 and IL-15 enhance the in vitro priming of naive CD4+ T cells for IFN-gamma but have differential effects on priming for IL-4. *J Immunol* 1996; **156**:2413–22.
37 Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 2003; **4**:1191–8.
38 Mueller DL, Seiffert S, Fang W, Behrens TW. Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells. A model for the long-term survival of memory cells. *J Immunol* 1996; **156**:1764–71.
39 Guarda G, Hons M, Soriano SF *et al.* 1-selectin-negative CCR7-effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. *Nat Immnol* 2007; **8**:743–52.
40 Marks-Konczalik J, Dubois S, Losi JM, Sabzevari H, Yamada N, Feigenbaum L, Waldmann TA, Tagaya Y. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc Natl Acad Sci USA* 2000; **97**:11445–50.
41 Riou C, Dumont AR, Yassine-Diab B, Haddad EK, Sekaly RP. IL-4 influences the differentiation and the susceptibility to activation-induced cell death of human naive CD8+ T cells. *Int Immunol* 2006; **18**:827–35.
42 Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* 2002; **2**:251–62.
43 Badovinac VP, Messingham KA, Jabbari A, Haring JS, Harty JT. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat Med* 2005; **11**:748–56.